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## 59. BLOOD NEUROPATHY TARGET ESTERASE AS BIOCHEMICAL MARKER FOR NEUROPATHIC ORGANOPHOSPHATES EXPOSURE

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### INTRODUCTION

Organophosphate-induced delayed neurotoxicity (OPIDN) is a distal degeneration of sensory and motor axons that occurs 2-5 weeks after an acute poisoning by several organophosphates (OPs) with antiesterase activity and appears as flaccid paralysis of the lower and, in the most severe cases, upper limbs [1,2]. This process is totally independent of inhibition of acetylcholinesterase (AChE) and can be induced by OPs with low acute toxicity [3,4]. OPIDN has occurred in epidemic proportions through the world [5]. The high human susceptibility to OPIDN [6], its insidious onset and usually permanent debilitating effects make the problem of monitoring of neuropathic OP exposure to humans as well the problem of an early diagnostics of OPIDN highly important. It is necessary to bear in mind that ability of some OPs along with acute cholinergic toxicity initiate OPIDN may to be of interest for different terrorist groups.

There is considerable evidence that a neuronal protein with serine esterase activity, neuropathy target esterase (neurotoxic esterase, NTE), is the primary target molecule in OPIDN. It is thought now that OPIDN is initiated by the organophosphorylation of NTE with a subsequent specific modification (aging) of the inhibited enzyme [3,7,8]. In the experimental animals OPIDN is associated with > 70% threshold of brain NTE inhibition after single exposures. The threshold in man is not known, although there are indications that it is similar.

The relationship between NTE inhibition/aging and development of OPIDN has potential to be further exploited as a biomarker [3,8,9]: inhibition of brain NTE within hours of exposure to OPs predicts potential for developing OPIDN in susceptible animal models (the adult hens) after delay in 1-5 weeks. NTE has also been found in circulating lymphocytes and platelets [10-12]. Lymphocyte NTE has been proposed for use as accessible biomarker of animals and human exposure to neuropathic OPs. [7,13-16]. Furthermore, the desirability of exploiting of such an accessible source of human NTE in health monitoring of exposed people has been discussed [6,13,17-19].

Whereas it should be noted that isolation of lymphocytes requires a highly equipped laboratory and highly skilled personnel; it is time-consuming process that provides a relatively low yield of cells for assay. In addition, NTE assay in lymphocytes requires a large sample of blood, and lymphocytes isolated are unstable under storage. This all restricts considerably the use of lymphocytes to monitor routinely NTE activity among individuals exposed to OPs in field and industrial conditions as well the carrying out epidemiological studies.

NTE is determined as that part of phenyl valerate hydrolysing activity which is resistant to inhibition by O,O-diethyl-4-nitrophenyl phosphate (paraoxon, non-neuropathic OP) but sensitive to inhibition by N,N'- di-iso-propyl phosphoro-amido fluoridate (mipafox, neuropathic OP) [3]. Phenol released as a result of phenyl valerate hydrolysis is usually

determined spectrophotometrically by measuring absorbtion of red coloring after the oxidative coupling phenol to 4-aminoantipyrine in alkaline potassium ferricyanide [20].

The application of spectrophotometry is often limited in biological samples, especially in samples with low NTE activity when their turbidity and possible presence of coloring of a sample reduce the accuracy of measurements, as well when only small quantity of biomaterial is accessible. The additional problem occurs when somebody works with blood. In this case the colored reaction product of phenol with 4-aminoantipyrine and potassium ferricyanide should be measured on the background of a red color of blood. The optical density of the 10-fold diluted homogenized whole blood at 492 nm (close to the optimum for the spectrophotometric phenol detection) was shown to be approximately 0.75-0.8 [21]. A dilution of blood cannot be effective because the sensitivity of the spectrophotometric NTE assay decreases sharply and becomes negligible at 100-fold dilution of the blood [21] that makes NTE analysis in whole blood completely impossible.

These problems could be eliminated with use of amperometric technique instead of spectrophotometry for phenol assay.

To create fast and simple methods of monitoring neuropathic OP exposure to humans suitable for epidemiological studies and early diagnostic of OPIDN we developed a new approach to analysis of NTE activity by means of tyrosinase-based biosensors. Such tyrosinase biosensors involve the enzymatic oxidation of phenol *via* catechol into o-quinone, the reaction proceeding with oxygen consumption [22]. The change in the concentration of phenol was monitored by oxygen uptake when the Clark-type oxygen electrode covered with tyrosinase immobilized in polyvinylalcohol used as an electrochemical transducer [23].

Electroreduction of quinone on a graphite electrode can also be used as a detection reaction for the quantification of phenol [24]. The tyrosinase carbon-paste biosensor for phenol provides electrochemical reduction of quinone to catechol directly at the electrode when the required potential (-50 mV) is applied (Fig. 1). This regenerating process amplifies the electrode response and makes it possible more sensitive phenol detection compared to the measurement of the oxygen consumption [21,23]. We showed that the use of tyrosinase carbon-paste electrode improves 10-fold the sensitivity of NTE activity determination in comparison with a spectrophotometric method and amperometric method with the Clark-type electrode modified by tyrosinase [21,23,25]. The developed tyrosinase carbon-paste electrode is characterized by high sensitivity and stability for phenol detection in a flow-injection mode. Time of analysis for a single phenol sample in flow mode was 2-3 min when the flow speed was 0.25 ml/min [21].

In the present work we report the results of studies of possibility of application of biosensor methods to the NTE activity analysis in whole blood as well the results of OPIDN modeling in experiments on animals with acute administration of the increasing doses neuropathic OP O,O-dipropyldichlorvinylphosphate (PrDChVP) and studying the correlation between NTE inhibition in brain and blood using spectrophotometric and biosensor methods

## MATHERIALS AND METHODS

*Chemicals:* Phenyl valerate (PV), mipafox (*N,N'*-diisopropylphosphorodiamido fluoridate), O,O-dipropyldichlorvinylphosphate (PrDChVP) were synthesized and characterized in the Institute of Physiologically Active Compounds Russian Academy of Sciences (Russia) [15] and in the Institute of Organic Chemistry Ukrainian Academy of Sciences (Ukraine). The purity of all substances was not less than 99% (by spectral, chromatographic and elemental analysis data). Mushroom tyrosinase (monophenol monooxidase, EC 1.14.18.1), activity 3800 U/mg for L-tyrosine, graphite powder, paraoxon (*O,O*-diethyl-4-nitrophenylphosphate), 4-aminoantipyrine, and potassium ferricyanide were

purchased from Sigma Chemical Co. (St. Louis, Missouri USA and Deisenhofen, Germany). Phenol was received from Merck (Darmstadt, Germany). A Coomassie protein kit was from Pierce (USA). All other chemicals were of analytical grade and used without further purification. Aqueous solutions were prepared using deionized water.

*Preparation of tyrosinase carbon-paste electrode and assembly of biosensor.* The tyrosinase electrode was prepared as described previously [21]. All measurements were performed with an applied potential of -50 mV vs. Ag/AgCl. The current was measured by means of a homemade amperometric detector "IPC2000" coupled to PC. The device operating and processing the results of electrochemical measurements were carried out with use of specially developed software.

## Tissues

*Neuronal NTE.* The lyophilized hen brain ($P_2+P_3$) membrane fraction preinhibited with paraoxon (40 µM, 45 min) was used as a source of NTE. It was prepared as described in [26,27] and stored in sealed ampoules. Before use, the ampoule content was suspended at 25°C with a glass/glass Potter homogenizer in 2 ml of a work buffer (50 mM Tris-HCl, 0.2 mM EDTA, pH 8.0). The preparations of neuronal NTE have a specific activity of about 40 nmoles phenyl valerate/min per mg of protein.

*Isolation of lymphocytes from human blood.* Fresh human blood stabilized by citrate with EDTA was used. Lymphocytes were isolated as described in [21,28] and homogenized in glass/glass Potter homogenizer before NTE assay.

*Blood preparation.* Whole human and hen blood stabilized by citrate with EDTA was homogenized in Potter glass/glass homogenizer, then diluted by the work buffer up to the desired level of dilution.

*Samples preparation for NTE activity determination by spectrophotometric and electrochemical methods.* NTE activity was determined according to the differential method of Johnson [20] as a microassay version [29] with slight modifications [30]. The diluted with work buffer samples of lymphocytes or the homogenized whole blood were incubated at 37°C with 50 µM of paraoxon for 20 min (sample B) or with 50 µM of paraoxon plus 250 µM mipafox for 20 min (sample C). Phenyl valerate then was added. After 30 min incubation at 37°C, the reaction was stopped by addition of aqueous SDS. Phenol released was assayed spectrophotometrically or amperometrically. In the case of the paraoxon-preinhibited preparation of the hen brain NTE the samples were incubated in work buffer (sample B) and in buffer with 250 µM mipafox (sample C) for 20 min at 37°C, following the incubation with phenyl valerate and reaction termination by SDS as described above.

*Spectrophotometric phenol assay.* Phenol released as a result of the enzymatic hydrolysis of phenyl valerate was assayed in 96-well microtiter plates at 492 nm using Microtiter Plate Reader SLT 340 ATTS (SLT Labinstruments GmbH, Germany) after the incubation of 100 µl of final reaction mixture at room temperature with 4-aminoantipyrine and potassium ferricyanide. Each measurement was made in triplicate. The absorbance difference between samples B and C was used for specific NTE activity calculation using the phenol standard calibration curve.

*Electrochemical phenol assay.* Prior to measurements, samples were diluted 10-50-fold in 0.1 M NaCl + 0.05 M sodium phosphate buffer, pH 7.0. Enzymatically released phenol was measured amperometrically after an injection of the diluted final reaction mixture into a flow of 0.1 M NaCl + 0.05 M sodium phosphate buffer, pH 7.0 *via* injector with 50 µl sample loop (Valve V-7, Pharmacia, Sweden). The flow rate was 0.25 ml/min. Each measurement was made in duplicate. The concentration of phenol was determined according

to the phenol calibration curve obtained under the same conditions. The difference in the analytical signals found in samples B and C was used for specific NTE activity calculation.

*Titration with inhibitors.* For titration curves obtaining and $I_{50}$ (the concentration of OP which inhibits 50% of a given enzyme activity under defined preincubation conditions) assessment a sample of enzyme was incubated with 10-12 different concentrations of studied OP from $10^{-9}$ to $10^{-3}$M for 20 min at 37°C, pH 8.0. The residual NTE activity was then determined, hydrolysis of phenyl valerate was stopped by aqueous SDS. Phenol released was assayed spectrophotometrically or amperometrically (see above). Each measurement was made in triplicate (for spectrophotometry) or in duplicate (for amperometry). $I_{50}$ values were calculated as described in [21]. Every value represents the mean ± SEM from 3 independent experiments.

*Protein assay.* Protein was determined using a Coomassie protein assay kit with bovine serum albumin as a reference standard.

*In vivo studies.*

*Animals:* Adult white Leghorn hens (18 months old, 1.5-2 kg) were from Noginsk poultry farm (Noginsk, Russia). Hens were kept 3 to a cage with food and water *ab libitum*. The hens were kept in room with 12-hr light cycle in which the temperature was controlled (20-23°C).

*(I) Inhibition of NTE in hen brain and lymphocytes in 24 hr after acute i.m. treatment with increasing doses of neuropatic OP O,O-dipropyldichlorvinylphosphate (PrDChVP).* PrDChVP was administered i.m. in doses 0.316, 0.4, 0.56, 1.0, 1.58, and 2.2 mg/kg to groups of 3 hens per every dose. All hens were pretreated with atropine sulfate, 20 mg/kg, 20 min before PrDChVP was administered. Control animals received atropine sulfate only. In 24 hr after PrDChVP administration hens were decapitated; blood was collected immediately in heparinized plastic vials, heparin was added to concentration 20E/ml, then blood was diluted 1.0/1.5 (v/v) with BSS (0.001% D-glucose, 5.0 mM $CaCl_2$, 98 mM $MgCl_2$, 14.5 mM Tris, 126 mM NaCl, pH 7.6).

*Lymphocytes* were isolated according to [14] by centrifuging in Ficoll-Verografin gradient density. NTE activity was determined spectrophotometrically in sonicated (10 min, power output 50W) lymphocytes by the Johnson differential method [20] using 40 min incubation with Phenyl Valerate.

*Brains* were rapidly removed; brain of every hen was homogenized at +4°C in 5 vol. of buffer (50 mM Tris-HCl, 0.2 mM EDTA, pH 8.0) with Potter homogenizer and centrifuged for 15 min at 9000 x g at +4°C. The brain 9S supernatant was used for NTE analysis [31].

*NTE activity in brain and lymphocyte samples* from hens treated with atropine and PrDChVP was determined by spectrophotometric method and compared to activity in tissue samples from animals treated with atropine only (control).

*Protein was assayed* by microbiuret methods with bovine serum albumin as a standard.

*(II) Inhibition of NTE in hen brain and blood in 24 hr after acute i.m. treatment with four increasing doses of PrDChVP.* PrDChVP was administered to hens i.m. in four doses have been chosen from the Experiment (I): 0.316, 0.4, 0.56, 1.0 mg/kg to groups of 3 hens per every dose. In 24 hrs after PrDChVP administration hens were decapitated, *blood* from every hen was collected immediately in a glass containing solution of 3,8% Sodium citrate and D-glucose (from the account 20 ml of anticoagulant per 100ml of blood), frozen in liquid nitrogen and stored at –20°C prior to NTE assay. After being allowed to thaw at room temperature the blood samples were used for NTE activity determination with biosensor method using the tyrosinase carbon-paste electrode.

*Brains* were rapidly removed, frozen in liquid nitrogen, weighted and stored at –20°C prior to NTE assay. After being allowed to thaw at room temperature they were used for 9S supernatant obtaining (see above) and spectrophotometric NTE activity determination.

*NTE activity in brain and whole blood samples* from hens treated with atropine and PrDChVP was determined and compared to activity in tissue samples from animals treated with atropine only (control). Linear regression analysis (Origin 5.0 software) was used to examine the correlation of NTE inhibition between tissues.

## RESULTS AND DISCUSSION

An excellent coincidence of the data on titration of hen brain NTE (paraoxon-pretreated lyophilyzed preparation) by mipafox obtained by both spectrophotometric and amperometric methods were found. The calculated $I_{50}$ are presented in the Table 1 in comparison with data described in the literature. Close values of $I_{50}$ for human lymphocyte NTE were also obtained when spectrophotometric and amperometric methods were used (Table 1). NTE activity in human lymphocytes measured amperometrically was found to be 14±3 nmoles/min per mg of protein (n=3) for freshly isolated lymphocytes that is in a good agreement with the values reported earlier [11,12,13,16]. Data obtained indicate to validity of measurements carried out with the developed biosensor.

To study a possibility of using biosensor technique for NTE measurement in whole blood an influence of biological materials on phenol assay was studied [21]. A number of phenol-like compounds that are present in biological samples can be substrates for the immobilized tyrosinase and the corresponding analytical responses cannot be ignored. It was found that contacts of the homogenized hen and human whole blood with the electrode surface do not leads to any notable contamination of the latter and only high content of biological material in analyte affected significantly the phenol assay. However, sensitivity of electrode was sufficient to measure phenol in 100-200-fold diluted samples for which the influence on the phenol signals is not so great [21].

A sensitivity of hen and human blood to non-neuropathic OP paraoxon inhibition was investigated with biosensor method and were found to be quite similar. According to data obtained, approximately 30% of a total phenyl valerate hydrolysing activity found in blood represent a paraoxon-resistant part both for hen and for human blood preparations. NTE activity in hen and human blood was found to be equal to 0.10±0.03 and 0.19±0.02 nmoles/min per mg of protein respectively.

Curves of NTE titration in hen and human blood by standard delayed neurotoxicant mipafox were shown to be close to those for neuronal and lymphocyte NTE. Data on $I_{50}$ for different preparations are summarized in the Table 1

Data obtained allow to conclude that the developed tyrosinase carbon-paste biosensor is suitable for NTE assay in whole human and hen blood when the usual spectrophotometric detection is impossible. The results look promising for using NTE activity in whole blood as biochemical marker of exposure to neuropathic OPs.

In order to use the measurement of blood NTE activity as a mirror of brain NTE, the correlation between the inhibition of the enzyme in brain and blood should be known. Assay of lymphocyte NTE was shown to provide a good monitor of exposure to axonotoxic OPs within 24 hr between exposure and measurement [14]. To study possibility of using blood NTE inhibition as biochemical marker of neuropathic OP exposure two series of experiments were carried out: the dependence between NTE inhibition in hen brain and lymphocytes as well in hen brain and blood was studied in 24 hr after acute i.m. injecting hens with increasing doses of model neuropathic OP O,O-dipropyldichlorvinylphosphate (PrDChVP). Data obtained in both series of experiments are presented in Table 2.

PrDChVP inhibited brain, lymphocyte and blood NTE in a dose-responsive manner (Fig.2).

There was good agreement between brain and lymphocyte NTE inhibition (Fig. 3, A) as well between brain and whole blood NTE inhibition (Fig. 3, B, C) and lymphocyte and whole blood NTE inhibition (Fig. 3, D). The data obtained suggest blood NTE activity as biochemical marker for neuropathic OPs exposure.

Taking into account a small volume and simplicity of blood sample preparation for biosensor NTE analysis as well as blood stability after freezing, the obtained results look promising for the development of systems for monitoring the occupational exposure of humans to neuropathic OPs, particularly, for in-the-field using, as well for epidemiological studies, and for development and improving the methods of early diagnostics of OPIDN.

## SUMMARY

NTE is a specific target for OPs that cause organophosphate-induced delayed neuropathy (OPIDN). The inhibition/aging of brain NTE within hours of exposure to OP predicts potential for the development of OPIDN in susceptible animal models. Lymphocyte NTE has also found some use as a biomarker of exposure to neuropathic OPs to man. Recently we developed a high-sensitive biosensor for the analysis of NTE as a combination of NTE catalyzed hydrolysis of phenyl valerate with phenol detection by a tyrosinase carbon-paste electrode. This biosensor was found to be suitable for NTE assay in whole human and hen blood when the usual spectrophotometric detection is impossible. NTE activity in hen and human blood was found to be equal to $0.10 \pm 0.03$ and $0.19 \pm 0.02$ nmoles phenyl valerate /min per mg of protein respectively. Sensitivity of hen and human blood NTE to standard delayed neurotoxicant mipafox were shown to be close to those for neuronal and lymphocyte NTE. Mipafox $I_{50}$ values for hen and human blood NTE were found to be equal $4.22 \pm 0.12$ and $6.27 \pm 0.43$ µM, respectively. To study possibility of using blood NTE inhibition as biochemical marker of neuropathic OP exposure the dependence between NTE inhibition in hen brain and lymphocytes as well in hen brain and blood was studied in 24 hr after acute i.m. injecting hens with increasing doses of model neuropathic OP O,O-dipropyldichlorvinyl phosphate (PrDChVP). PrDChVP was shown to inhibit brain, lymphocyte and blood NTE in a dose-responsive manner. There was good agreement between brain and lymphocyte NTE inhibition as well between brain and blood NTE inhibition, and lymphocyte and blood NTE inhibition that suggest blood NTE activity as biochemical marker of neuropathic OPs exposure. Taking into account small volume and simplicity of blood sample preparation for biosensor NTE analysis the obtained results look promising for the development of systems for monitoring the occupational exposure of humans to neuropathic OPs, particularly, for in-the-field using, as well for epidemiological studies, and for development and improving the methods of early diagnostics of OPIDN.

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**KEYWORDS:**

## FIGURES AND TABLES.

Fig1. Catalytical oxidation of phenol in the presence of tyrosinase
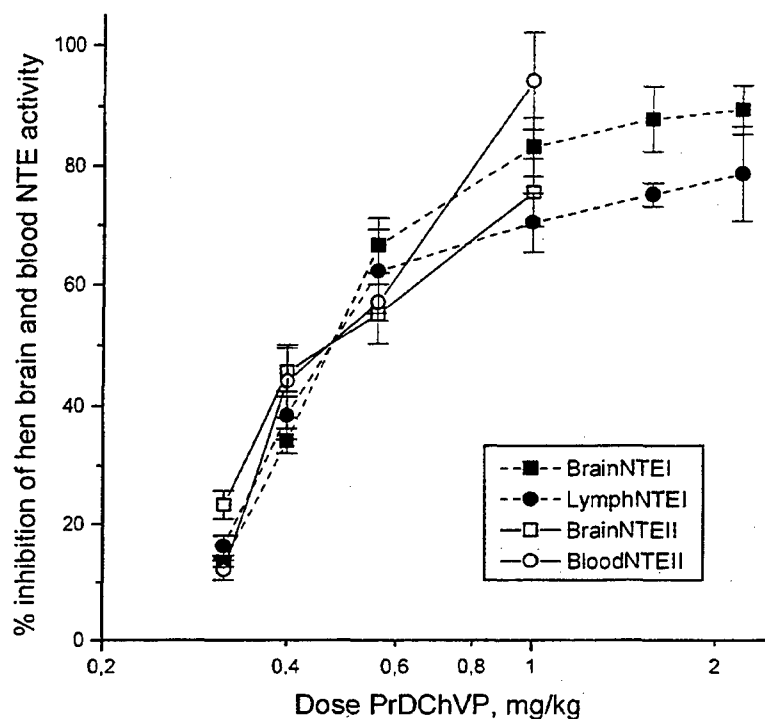


Fig.2. Dose-related NTE inhibition in brains, lymphocytes and whole blood of hens given neuropathy-inducing organophosphorus compound O,O-dipropyldichlorvinyl phosphate (24 hr after exposure). Results are expressed as means ± SEM, n=3. Brain (---■---) and lymphocyte (---●---) NTE from the experiment (I); brain (—□—) and blood (—○—) NTE from the experiment (II).
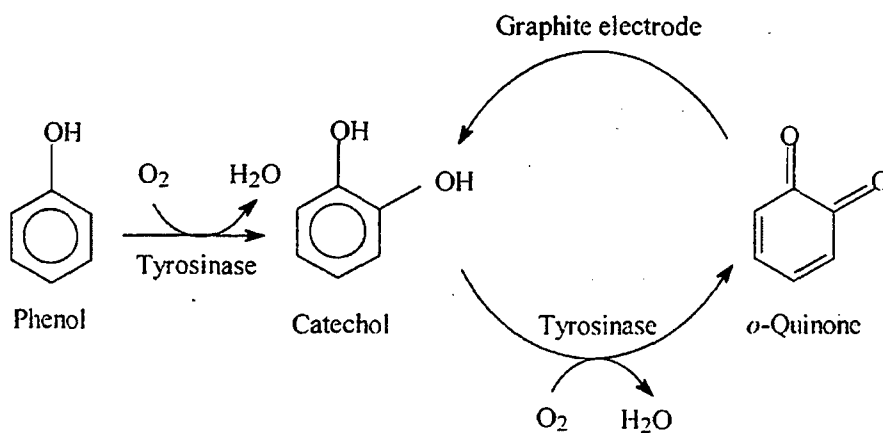
Fig. 3. A - correlation between hen brain and peripheral lymphocyte NTE inhibition (exp. I) (R = 0.991, SD = 5.02, N = 4, P = 0.00857); B – correlation between hen brain and whole blood NTE inhibition (exp. II) (R = 0.997, SD = 1.93, N = 4, P = 0.00267); C – correlation between brain (the mean from experiments I and II) and whole blood NTE inhibition (R = 0.982, SD = 6.11, N = 4, P = 0.01825); D – correlation between hens lymphocyte (I) and whole blood (II) NTE inhibition (R = 0.946, SD = 9.73, N = 4, P = 0.05444).

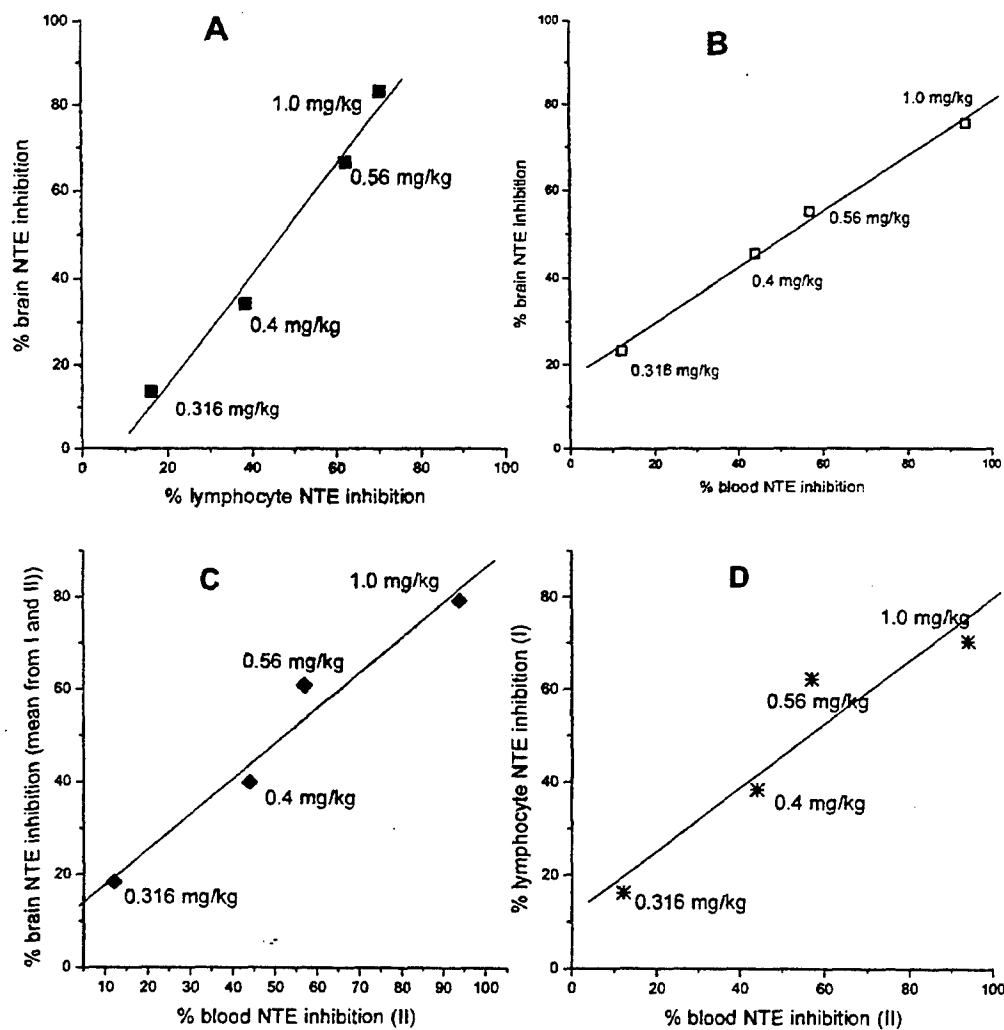Table 1. Mipafox $I_{50}$ values determined spectrophotometrically or amperometrically for NTE activity from different sources

| Source | $I_{50}$ mipafox, µM (microM) amperometry | $I_{50}$ mipafox, µM colorimetry | $I_{50}$ mipafox, mM literature data |
|---|---|---|---|
| Hen brain | $4.32 \pm 0.28$ | $4.20 \pm 0.54$ | $3.80 \pm 0.78$ [27]; 7 [10,33] |
| | | | $7.3 \pm 0.8$ [34]; 3.1 [35] |
| Hen whole blood | $4.22 \pm 0.12$ | | |
| Human lymphocytes | $8.38 \pm 0.88$ | $7.58 \pm 0.79$ | $9.6 \pm 0.8$ [13] |
| Human whole blood | $6.27 \pm 0.43$ | | |

" Each value represents the mean ± SEM for at least 3 independent experiments.

Table 2. Inhibition of Neuropathy target esterase (% from control) in brains, peripheral lymphocytes and whole blood of hens dosed with PrDChVP. (I) – results of the first series of experiments, (II) – results of the second series of experiments.

a) NTE inhibition in brain, lymphocytes and blood presented as the mean ± SD (n=3). Every measurement was made in duplicate. Control activities: (I) hen brain NTE = $30.9 \pm 2.8$ nmol PheVal/min/mg protein, hen lymphocyte NTE = $9.0 \pm 1.4$ nmol PheVal/min/mg protein; (II) hen brain NTE (brain after freezing) = $4.21 \pm 0.22$ nmol PheVal/min/mg protein, Hen blood NTE = $0.107 \pm 0.013$ nmol PheVal/min/mg protein

| Dose, mg/kg | (I) | | (II) | |
|---|---|---|---|---|
| | Brain NTE inhibition, % [a] | Lymphocyte NTE inhibition, % [a] | Brain NTE inhibition, % [a] | Blood NTE inhibition, % [a] |
| 0,316 | 13,5±0,8 | 16,3±1,7 | 23,3±2.4 | 12,1±1,7 |
| 0,4 | 34,1±2.1 | 38,4±4,1 | 45,5±4,0 | 44,0±6,1 |
| 0,56 | 66.5±4,6 | 62,2=7,0 | 55,1±4,9 | 57,0±3,0 |
| 1 | 83,1±4,9 | 70,3±5,1 | 75,4±5,7 | 94,0±8.0 |
| 1,58 | 87,7±5,4 | 75,0±2,0 | - | - |
| 2,2 | 89,2±4,0 | 70,3±7,9 | - | - |